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Application of:	Michael A. Zeligs	Confirmation No.:	9606
Serial No.:	10/616,477	Art Unit:	1618
Filed:	July 9, 2003	Examiner:	Ebrahim, Nabila G
For:	Phytochemicals for the Treatment of Mastalgia, Endometriosis and HPV-related Conditions Including Cervical Dysplasia	Attorney Docket No:	9439-015-999

DECLARATION OF VALERIE HEITCHEW UNDER 37 C.F.R. 1.131

Mail Stop AF
Commissioner for Patents
P.O. Box 1450
Alexandria, VA 22313-1450

Sir:

I, Valerie Heitchew, do declare and state that:

1. I am a citizen of the United States residing at 484 Battlefield Bluff Drive, New Market, Virginia, 22844.

2. I am the patient "V.H." whose treatment for cervical dysplasia is described in Example 13 of U.S. Patent No. 6,689,387.

3. Prior to late-1998, in addition to being troubled with chronic breast pain, I had a history of abnormal Pap Tests which periodically showed abnormal cervical cells starting in my thirties.

4. Late in 1998, Dr. Zeligs contacted me to see if I would participate in preliminary use of topical creams and capsules which contained Diindolylmethane (DIM). I was aware of Dr. Zeligs' research and development efforts using natural indoles, such as DIM, and agreed to serve as a volunteer to use the DIM preparations and continue my routine visits with my personal health care providers.

5. I recall that in July, 1998 that a Pap Test done by my primary physician, Dr. John Stauffer, showed more highly abnormal cells than previous tests and that these cells were indicative of early cervical dysplasia. I was closely following this condition

because I was advised that I would need surgical intervention if the condition did not improve.

6. In January-February 1999, I began to use the topical breast cream provided by Dr. Zeligs with good results for my breast pain. This was followed by the use of Indolplex™ (BioResponse's-DIM) capsules which I preferred because they were more convenient than the cream. The capsules were provided to me by BioResponse.

7. Following the use of the Indolplex™ capsules, I noted resolution of chronic vaginal discharge, which had been a recurrent symptom. Prior to this time, I had associated the presence of this discharge with abnormal pap test results.

8. I recall returning to Dr. Stauffer on August 3, 1999 and undergoing a repeat gynecologic examination. At the time of this examination, I was told by Dr. Stauffer that my cervix had a much more normal appearance on visual examination and during my repeat Pap Test.

9. I recall reporting this important improvement to Dr. Zeligs in a phone conversation within one or two days of my August 3, 1999 gynecologic examination.

10. I recall receiving a phone report from Dr. Stauffer office shortly after my August 3, 1999 gynecological examination, which provided the results of my Pap Test, confirmed my improvement, and indicated that I would not be needing surgery.

11. I declare further that all statements made in this Declaration of my own knowledge are true and that all statements made on information and belief are believed to be true and further that these statements are made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the application or any patent issuing thereon.

Valerie Heitchew 9/14/07

Date: Valerie Heitchew

Express Mail No.: EV 473 971 634 US

Date Mailed: January 25, 2007

Application No.: 10/616,477 Filed: July 9, 2003

Inventor: Michael A. Zeligs

For: **PHYTOCHEMICALS FOR THE TREATMENT OF MASTALGIA, ENDOMETRIOSIS AND HPV-RELATED CONDITIONS INCLUDING CERVICAL DYSPLASIA**

The stamp of the Patent Office hereon may be taken as an acknowledgment of the date stamped of the following:

(1) Amendment Under 37 C.F.R. 1.111;
(2) Amendment Fee Transmittal Sheet (in duplicate);
(3) Petition for Extension of Time (in duplicate);
(4) Supplemental Information Disclosure Statement Under 37 C.F.R. §1.56 and §1.97 (in duplicate);
(5) List of References Cited by Applicant, accompanied by a copy of references C21-C23.

File No. : 9439-015-999 Sender: TEF/DJK

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MODIFICATION OF CARCINOGEN METABOLISM BY INDOLYLIC AUTOLYSIS PRODUCTS OF *BRASSICA OLERACEAE*

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ABSTRACT

Cruciferous plant foods contain large quantities of secondary plant metabolites that have been shown to inhibit chemically induced carcinogenesis in animals. One mechanism by which these chemicals may inhibit carcinogenesis is through the induction of enzymes, such as cytochrome P-450-dependent monooxygenases, glutathione S-transferases (GST) or epoxide hydrolases (EH), which metabolize carcinogens to more polar and excretable forms. Cruciferous vegetables of the *Brassica* genus (*e.g.* Brussels sprouts, cauliflower, broccoli) contain μg/g levels of an indolylmethyl glucosinolate commonly known as glucobrassicin. Upon disruption of the plant material, as in food preparation or chewing, a thioglucosidase-mediated autolytic process ensues generating indole-3-carbinol (I3C), glucose, and thiocyanate ion. At acid pH comparable to that found in the stomach, I3C forms a wide variety of condensation products ranging from linear and cyclic dimers, trimers and tetramers to extended heterocyclic compounds such as indolocarbazoles. Experiments reviewed here indicate that these indole-condensation products are the compounds responsible for some of the alterations in carcinogen metabolism observed in animals fed either I3C or any of several *Brassica* plant foods.

ANTICARCINOGENIC PROPERTIES OF *BRASSICA* VEGETABLES

The National Research Council, Committee of Diet, Nutrition, and Cancer has recommended increased consumption of vegetables of the *Brassica* genus as a measure to decrease the incidence of human cancer (National Research Council, 1982). This recommendation is based on epidemiological evidence (Graham, 1983) and results from animal experimentation (Stoewsand *et al.*, 1978; Wattenberg, 1983) that suggest that these vegetables possess cancer-inhibiting properties. The committee's review of the scientific literature led to the suggestion that the inhibitory effects of these vegetables may be related to the presence of a number of nutritive and nonnutritive constituents known to inhibit chemically induced carcinogenesis in experimental animals.

Stoewsand *et al.* (1978) published the first study of an anticarcinogenic effect of *Brassica* vegetables. In this study, rats that were exposed to the hepatocarcinogen aflatoxin B_1 and fed on purified diets supplemented with 25% cauliflower lived considerably longer and had smaller

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tumors than did the unsupplemented controls. Results of further experiments showed that diets high in cabbage had similar anticarcinogenic properties (Boyd *et al.*, 1982). The anticarcinogenic properties of these vegetables were also shown in a study by Wattenberg (1983) in which rats fed on a diet supplemented with 10% cabbage or cauliflower had fewer mammary tumors induced by dimethylbenzanthracene than did the unsupplemented controls.

Wattenberg (1983) has suggested that tumorigenesis is inhibited by carcinogen-metabolizing systems induced by compounds in *Brassica* plants. This hypothesis proposes that minor dietary constituents function as anticarcinogenic substances by virtue of their ability to enhance the activities of xenobiotic-metabolizing enzymes that shunt the metabolism of precarcinogenic substrates through detoxification pathways rather than through pathways leading to genotoxic species capable of initiating neoplasia. Additionally, agents that induce xenobiotic-metabolizing enzymes could also elicit anticarcinogenic effects *via* increases in presystemic metabolism of a carcinogenic substrate, thereby decreasing the dose of carcinogen reaching target tissues (Wattenberg, 1970).

Supporting this hypothesis is the observation that many inducers of xenobiotic-metabolizing systems have an inhibitory effect on a variety of chemically induced neoplasias. Early reports of this relationship include the demonstrations that administration of 1,2,5,6-dibenzofluorene inhibited 3-methylcholanthrene-induced tumors (Lacassagne *et al.*, 1945; Riegel *et al.*, 1951) and that 3-methylcholanthrene inhibited 3'-methyl-4-dimethylaminoazobenzene-induced tumors (Richardson *et al.*, 1951; Conney *et al.*, 1956). Since these early reports, results from a number of studies have supported the concept that induction of xenobiotic-metabolizing enzymes can lead to decreased tumor yields resulting from exposure to carcinogenic agents. For example, phenobarbitone inhibits aflatoxin-induced hepatocarcinogenesis in rats (McLean and Marshall, 1971); DDT inhibits dimethylbenzanthracene-induced mammary tumors and leukemia in rats (Silinskas and Okey, 1975); 3-methylcholanthrene inhibits dimethylbenzathracene-induced mammary tumors in rats (Wheatly, 1968); and β-naphthoflavone inhibits benzo[a]pyrene-induced skin tumors in mice (Wattenberg and Leong, 1970).

Although it is known that many of the same xenobiotic-metabolizing systems involved in the detoxification of carcinogenic compounds can also be involved in their bioactivation (Gelboin, 1980), the relationship between inducing agents and carcinogenic outcome is not well understood. Monooxygenases, EHs, and GSTs are also involved in the bioactivation of certain chemical carcinogens *in vitro* (Rannug *et al.*, 1978; Schmassmann and Oesch, 1978; Wood *et al.*, 1976). Additionally, many inducers of xenobiotic-metabolizing systems, such as phenobarbital and 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD), are promoters of carcinogenesis (Pitot, 1982; Pitot *et al.*, 1980). Thus, induction of xenobiotic-metabolizing enzymes may explain decreased tumor yield, but does not necessarily indicate that exposure to inducing agents is a general prescription for cancer prophylaxis.

DIETARY EFFECTS ON XENOBIOTIC METABOLISM

Early reports by Brown *et al.* (1954) and Wattenberg (1970) began to characterize the modification of xenobiotic metabolism by dietary constituents, including the description of increases in hepatic methyl-4-dimethylaminoazobenzene demethylase activity and extrahepatic aryl hydrocarbon hydroxylase (AHH) activity in rodents fed on commercial chow formulations. The presence of significant quantities of inducing agents in *Brassica* vegetables was first demonstrated by Wattenberg (1972) who noted the ability of a number of *Brassica* vegetables to induce extrahepatic AHH

activity. Later, Sparnins *et al.* (1982) noted the effects of these vegetables on GST activity in rodents. Babish and Stoewsand (1975) and Stoewsand *et al.* (1978) described increases in hepatic aminopyrine N-demethylase N-methylaniline, N-demethylase, and p-nitroanisole O-demethylase in rats fed on cabbage or cauliflower. More recent reports have expanded upon these results by demonstrating that consumption of broccoli, cabbage, or Brussels sprouts leads to the induction of hepatic and intestinal microsomal and cytosolic EH and quinone reductase activities in rodents (Aspry and Bjeldanes, 1983; Hendrich and Bjeldanes, 1983; Hendrich and Bjeldanes, 1986; Bradfield *et al.*, 1985; Salbe and Bjeldanes, 1985; Salbe and Bjeldanes, 1986).

Dietary modification of xenobiotic metabolism has also been shown to alter the biological fate of therapeutic agents in rats and humans. Pantuck *et al.* (1976) showed that rats fed on cabbage or Brussels sprouts had increases in the oxidative metabolism of phenacetin and hexobarbital. Similar effects were observed on the oxidative metabolism of the analgesics phenacetin and aminopyrine in human subjects fed these vegetables at 500 g/d (Pantuck *et al.*, 1979). In another human study, these dietary treatments increased the clearance rate of acetaminophen in male subjects. The increase in clearance rate appeared to be the result of an increased capacity to form acetaminophen glucuronides, rendering this drug more readily excretable (Pantuck *et al.*, 1984).

IDENTIFICATION OF INDUCERS OF XENOBIOTIC-METABOLIZING ENZYMES FROM *BRASSICA OLERACEA*

The knowledge that cruciferous vegetables can inhibit chemically induced carcinogenesis and induce a variety of xenobiotic-metabolizing enzymes has led to attempts to isolate the constituents responsible for these properties. The relative ease of quantitating effects on enzyme activity as opposed to measuring inhibition of neoplasia, as well as the correlation between potency as inducing agents and inhibition of chemical carcinogenesis, has led to efforts to isolate compounds that modify xenobiotic-metabolizing enzymes. Fenwick *et al.* (1983) have provided the groundwork to isolating these compounds by establishing the biochemical levels and identities of a series of secondary plant metabolites, known as glucosinolates.

Glucosinolate levels in some cultivars of the genus *Brassica* are reported to be as high as 180 mg/g (Anand, 1974). Levels of glucosinolates are known to be dependent on soil nitrates, sulfates, conditioners, irrigation, growing season, cultivar, and crop spacing (Heaney and Fenwick, 1981; Heaney *et al.*, 1983; Miller *et al.*, 1983; Bible *et al.*, 1980). The generalized structure for glucosinolates is shown in Figure 1. All glucosinolates contain β-D-thioglucose and sulfate moieties (Ettlinger and

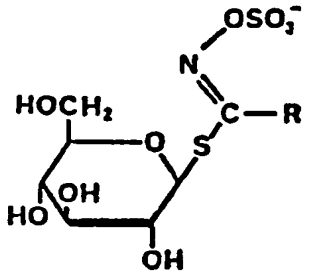

FIGURE 1. GLUCOSINOLATE STRUCTURE

Kjaer, 1968). The structure of the R-group is derived biosynthetically from amino acids (Kjaer and Olesen-Larsen, 1973). To date, over 70 unique glucosinolates have been characterized and identified in plants throughout the order of Capparales (Kjaer and Olesen-Larsen, 1976). This order contains the family *Cruciferae*, which in turn includes a number of commonly consumed plants, *e.g.* cole crops (Brussels sprouts, broccoli, cauliflower, kale, cabbage, Kohl-rabi *Brassica oleracea*), condiments (white mustard; *Brassica hirta*), radish (*Raphanus sativus*), papaya (*Carica papaya*) and forages (rapeseed; *Brassica napus*) (Fenwick *et al.*, 1983).

In addition to high levels of glucosinolates, plants of the *Cruciferae* contain high levels of thioglucosidase (EC 3.2.3.1) commonly referred to as myrosinase (Pihakaski and Pihakaski, 1978). Thioglucosidase appears as a group of isozymes which have a broad substrate specificity for all glucosinolates (Bjorkman and Lonnerdal, 1973). The thioglucosidase is localized in dilated cisternae of the rough endoplasmic reticulum. Although the exact cellular localization of glucosinolates has not been determined, this substrate does appear to reside in a compartment separate from the thioglucosidase (Pihakaski and Iversen, 1976). When the cell's integrity is disrupted, as in chewing or food preparation, enzyme and substrate come together. The generalized autolysis reaction generates glucose, sulfate, and aglucones (Ettlinger and Lundeen, 1957). The nature of the aglucone generated is dependent on the structure of the R-group, the pH at which the hydrolysis is carried out, and the presence of enzymatic modifiers such as ascorbic acid (Virtanen, 1965); Ettlinger *et al.*, 1961).

The role these thioglucosides play in plant physiology is unclear, although reports suggest a number of allelopathic effects, including insecticidal activity towards herbivorous predators (Blau *et al.*, 1978), inhibitory effects on growth and germination of competitive grasses (Kutacek, 1964; Leblova-Svobodova and Kostir, 1962), as well as functioning as an inactive storage form of plant growth hormones (Skytt Anderson and Muir, 1966). Biological effects of glucosinolates in animals include the goitrogenic activity of the autolytic products 5-vinyl-thiooxazolidinethione (goitrin) and thiocyanate ion (VanEtten and Wolff, 1973) and the hepatotoxicity and nephrotoxicity of autolytically generated epithiobutanes (Gould *et al.*, 1980).

INDOLYLIC METABOLITES, INHIBITION OF NEOPLASIA AND INDUCTION OF DRUG-METABOLIZING ENZYMES

Using the induction of AHH activity as a bioassay, I3C, indole-3-acetonitrile (IAN), indole-3-carbaldehyde (I3CHO) and 3,3'-diindolylmethane (I33') were isolated from an active fraction of an extract of *Brassica oleracea* (Loub *et al.*, 1975). These indoles are products of the thioglucosidase-mediated autolysis of indolylmethyl glucosinolate (also known as glucobrassicin (Virtanen, 1965) (Figure 2). Testing of these purified compounds demonstrated that I3C, IAN and I33' induced monooxygenases and/or GSTs (Sparnins *et al.*, 1982; Loub *et al.*, 1975). Additionally, I3C and I33' inhibited dimethylbenzanthracene-induced mammary tumors, and all three inhibited benzo[a]pyrene-initiated forestomach neoplasia in mice (Wattenberg and Loub, 1978). Although extensive comparisons were not performed, these early studies indicated that I3C was the isomer with the greatest biological potency as an inducer of monooxygenase activity and an inhibitor of neoplasia.

In our early studies of the pharmacology and chemistry of these compounds, the high sensitivity of I3C to acidic media became obvious. IAN and I33' were much less sensitive than was I3C. Subsequently, we found that I3C's chemical reactivity lies at the heart of its biological reactivity.

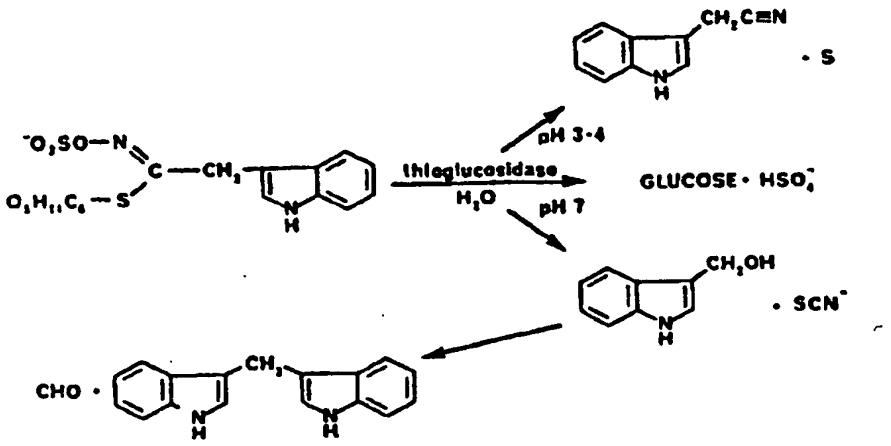

FIGURE 2. AUTOLYSIS OF GLUCOBRASSICIN

I3C and related indoles induce a variety of xenobiotic-metabolizing enzymes, most notably, cytochrome P-$_1$-450-dependent monooxygenases, such as AHH and ethoxyresorufin O-deethylase (Loub *et al.*, 1975; Shertzer, 1982; Bradfield and Bjeldanes, 1987). These isozymes are regulated by a soluble ligand-responsive transcription factor, the Ah receptor. This receptor acts by binding ligand, interacting with genomic elements and increasing the transcriptional rates of the genes that encode these enzymes (*e.g.* the cytochrome P$_1$450 gene, Whitlock, 1987). Agonists of the Ah receptor include a variety of planar aromatic hydrocarbons, such as benzo[a]pyrene, 8-napthoflavone and 3-methylcholanthrene, as well as halogenated dibenzo-p-dioxins, biphenyls and azobenzenes. The structure-activity relationships for agonists of this receptor suggest that for nonhalogenated agonists, there is a requirement for extended planarity, and at least three aromatic rings for agonist binding (Piskorska-Pliszczynsk *et al.*, 1986; Poland, personal communication). I3C does not meet this last criterion and on a structure-activity basis appears to be an unlikely agonist of the Ah receptor. In fact, Gillner *et al.* (1985) and Poland (personal communication) have shown that I3C did not bind to the Ah receptor isolated from the rat or mouse.

The fact that I3C does not possess the structural features of an Ah receptor ligand, yet initiates biological responses characteristic of a receptor agonist suggested to us that I3C was altered *in vivo* ("bioactivated") to a form capable of receptor binding. A number of recent studies now implicate gastric acidity as the catalyst for this bioactivation and provide some clues as to the structure of the generated agonists. Evidence to suggest a role of gastric acidity in the bioactivation of I3C include:

1) results from structure-activity studies with simple indoles that describe a correlation between the instability of indoles in acidic solution and their potency as inducers of monooxygenase activity (Bradfield and Bjeldanes, 1987);

2) the observation that I3C is biologically active when administered orally, yet inactive when administered intraperitoneally (a route that allows the indole to bypass the acidity of the stomach) (Shertzer, 1982; Bradfield and Bjeldanes, 1987);

3) the fact that products generated by exposure to I3C to acidic solution are biologically active by either the intraperitoneal or oral routes;

4) the demonstration that I3C and an equivalent mass of its acid generation products have equivalent biological potency when administered orally (Bradfield and Bjeldanes, 1987).

Initial fractionation studies with I3C acid products suggested that a number of different condensation reactions occur and that many of the products possess biological activity (Bradfield and Bjeldanes, 1987). Recently, structures of some of these products have been determined. Among the products generated by treatment of I3C with acidic solution is a series of linear and cyclic methyleneindole trimers and tetramers, as well as indolocarbazoles (Figure 3). Preliminary results indicate that all these products have demonstrable binding affinity for the Ah receptor and elicit biological responses indicative of classical receptor agonists (Bradfield and Bjeldanes, manuscript in preparation). The susceptibility of substituted 3-hydroxy-methylindole derivatives to acid catalyzed elimination reactions is entirely consistent with the reactivity expected for vinylogous carbinolamines of this type. Thus, participation of the nonbonding electrons on nitrogen facilitates elimination of substituents on the α-carbon (for carbinolamines) or the δ-carbon (for vinylogous carbinolamines). The initial product of elimination of water from I3C is presumably 3-methyleneindolenine which by self condensation can produce the isolated dimeric and oligomeric products (Figure 3).

The classic studies by Virtanen (1965) on the autolysis of glucobrassicin indicated that at neutral pH (such as chewing or food preparation), I3C is the major product and can condense to I33' or react with ascorbic acid to generate ascorbigen. Our studies confirm the generation of I3C as the major autolysis product both in macerated plant material and in an *in vitro* system composed of thioglucosidase and purified glucobrassicin. Our experiments indicate that autolysis can occur even in plant material that has been boiled for 5 minutes (Bradfield and Bjeldanes, 1987B). These results suggest that I3C is likely to be found in cooked, processed, as well as in uncooked *Brassica* vegetables.

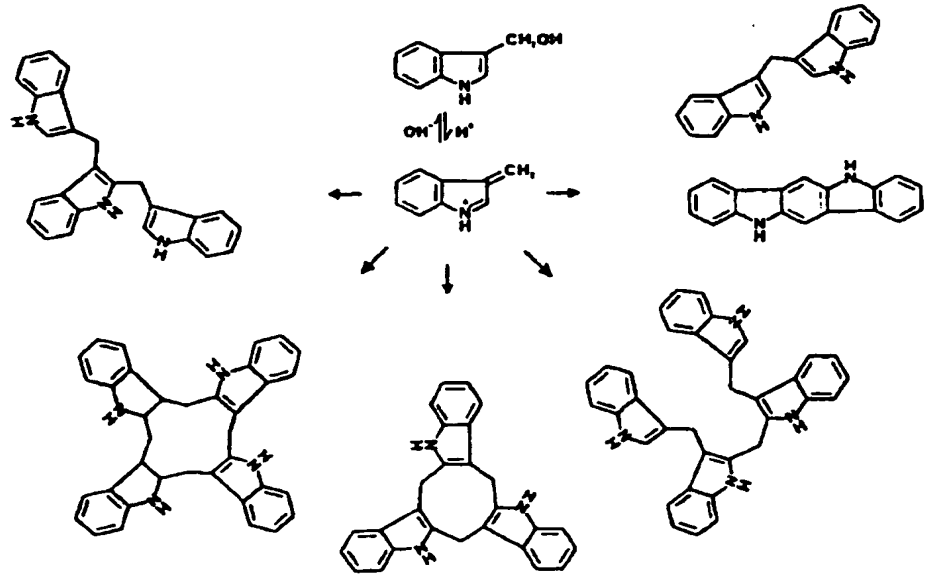

FIGURE 3. ACID CONDENSATION PRODUCTS OF I3C

The instability of I3C has important implications in assessing its dietary relevance. Our data suggest that the half-life of I3C in macerated plant material is approximately 12 hours at room temperature (Bradfield and Bjeldanes, 1987B) and that levels of I3C are substantially reduced by oxidative processes during the drying of plant material (Bradfield and Bjeldanes, 1987B, 1987C). Therefore, experiments that have utilized dried plant material or extended autolysis times may have generated levels of I3C, IAN, I3CHO, and I33' substantially lower than those levels generated during human consumption of fresh or cooked vegetables.

The National Research Council Report suggests that IAN, and not I3C, is the major autolysis product generated in *Brassica* vegetables (National Research Council, 1982). We believe that suggestion is incorrect for the following reasons:

1) HPLC analysis of the levels of I3C and IAN in freshly macerated plant material indicates that I3C is the major autolysis product and IAN is a minor one);
2) IAN has little biological potency as an inducer of monooxygenase activity (Loub *et al.*, 1975; Bradfield and Bjeldanes, 1987; Shertzer, 1982) and is the weakest indolic inhibitor of neoplasia (Wattenberg and Loub, 1978);
3) the actions of I3C, but not IAN, (Bradfield and Bjeldanes, 1987) are mediated through acid condensation products that are agonists of the Ah receptor; and
4) many agonists of the Ah receptor (*e.g.*, chlorinated dibenzo-p-dioxins and dibenzofurans) are among the most carcinogenic, teratogenic, and acutely toxic compounds known (Poland and Knutson, 1980).

The relationship between agonist activity and toxic effects suggests that consumption of large quantities of *Brassica* vegetables or their indolic constituents should be avoided.

CONCLUSION

The generation of Ah receptor agonists *via* the condensation of I3C in the acidic contents of the stomach appears to explain the mechanism by which I3C elicits induction of many xenobiotic-metabolizing enzymes. At present, it is not clear whether these condensation products also account for the anticarcinogenic activity of 3-substituted indoles and of *Brassica* vegetables. If, in fact, I3C acid condensation products bind to the Ah receptor as do highly toxic compounds, such as TCDD, we should thoroughly understand the toxicology of 3-substituted indoles before recommending them as desirable components of a diet to reduce the incidence of human cancer.

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Oligomerization of Indole-3-carbinol in Aqueous Acid

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Indole-3-carbinol [I3C, also called 3-(hydroxymethyl)indole] is a naturally occurring modulator of carcinogenesis with a biological activity that is at least partially dependent on its conversion to active substances in acidic media. We compared the identities of the major oligomeric products of I3C produced under conditions approximating those found in gastric juice with the reported identities of products of 3-substituted indoles produced under enzymatic and other nonenzymatic conditions. After a 10-min treatment in aqueous HCl solution, I3C was converted in 18% yield to a mixture of acetonitrile-soluble products, the major components of which (as determined by HPLC) were diindol-3-ylmethane (5.9%), 5,6,11,12,17,18-hexahydrocyclononal[1,2-*b*:4,5-*b'*:7,8-*b''*]triindole (2.0%), and [2-(indol-3-ylmethyl)indol-3-yl]indol-3-ylmethane (5.9%). Tentative assignments were made for 3,3-bis(indol-3-ylmethyl)indolenine (0.59%), a symmetrical cyclic tetramer (0.64%), and a linear tetramer (1.1%). Indolo[3,2-*b*]carbazole (ICZ) was formed slowly in aqueous acidic solutions in low yields (2.0 ppm) which increased to greater than 90 ppm following addition of an organic solvent [tetrahydrofuran (THF) or dimethylformamide (DMF)] to a neutralized solution. Relative yields of trimers vs dimer increased with decreasing pH and with decreasing starting concentration of I3C. Evidence is presented that ICZ formation may not involve radical intermediates as is characteristic of photodynamic processes. A mechanistic rationale is presented for the formation of the identified products.

Introduction

Indole-3-carbinol [I3C;[1] also called 3-(hydroxymethyl)-indole] is an autolysis product of a glucosinolate, glucobrassicin, which occurs in *Brassica* vegetables such as cabbage, broccoli, and Brussels sprouts. I3C is a potent inducer of cytochrome P-4501A1-dependent monooxygenase (CYP1A1) and can modulate the potency of carcinogens. When administered before a carcinogen, I3C can act as an inhibitor of cancer initiation, most likely by altering carcinogen metabolism (*1*). If administered after a carcinogen, I3C can act as a promoter of carcinogenesis (*2*). I3C also modifies estrogen metabolism in humans and is under study as a protective agent against mammary cancer (*3*). This dual cancer-modulating activity is generally not seen in substances of small molecular weight such as I3C but is a characteristic of certain polycyclic aromatic substances, most notably 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) (*4*).

An explanation for this unusual activity of I3C arises from the observations that I3C is highly sensitive to comparatively mild acidic conditions (*5*) and that under these conditions I3C is converted to biologically active substances (*6*). We reported previously that among these substances is indolo[3,2-*b*]carbazole (ICZ, Scheme I) which, in contrast with I3C, is a potent inducer of CYP1A1 without contact with acid and has a high affinity for the *Ah* receptor protein (*7*). The *Ah* receptor is a control factor for the synthesis of CYP1A1 and binds with high affinity to TCDD (*8*).

For the present study we investigated the identities of the major oligomeric products of I3C produced in aqueous acid and the effects of reaction conditions on the composition of this mixture. Our purpose was to gain insight into the chemical processes by which I3C is converted into biologically active substances under aqueous acidic conditions approximating those found in gastric juice.

Experimental Section

General Methods. We purchased I3C from Aldrich Chemical Co. (Milwaukee, WI) as the hydrate and purified it by recrystallization from toluene. Electron impact mass spectra and 400-MHz proton NMR spectra were obtained from the mass spectrometer and the NMR laboratories of the College of Chemistry, University of California at Berkeley.

HPLC Methods. For all the HPLC analyses we used a C-18 bonded-phase column (Beckman Ultrasphere-ODS, 4.6 × 250 mm, San Ramon, CA. The mobile-phase solutions were 60–65% acetonitrile in water or in 31 mmol/L ammonium dihydrogen phosphate adjusted to pH 6.7 with aqueous ammonia. Typically, the sample volumes were 0.01 mL, and the column flow rate was 1 mL/min. The detector was either an Altex/Beckman UV/vis spectrophotometer, Model 155-40, set at 280 nm or a Perkin-Elmer Model LS-4 fluorescence spectrometer set for excitation at 335 nm and emission at 405 nm. We received the HPLC–MS analyses with the help of I.-S. Kim (California Department of Public Health, Berkeley, CA) using a Hewlett-Packard Model 5988 system with a particle beam interface between the microbore column and MS detector.

Preparation of ICZ. Following the procedure of Robinson (*9*), we prepared ICZ by the Fischer indolization of the bisphenylhydrazone of cyclohexane-1,4-dione [1,4-bis(phenyl-1,1-diazenediyl)cyclohexane] with a mixture of glacial acetic acid and concentrated sulfuric acid as the catalyst. Our values for mass spectrum or UV spectrum of the synthesized material were identical to literature values (*9*, *10*). Also identical were chromatographic retention values and peak shape characteristics (using the HPLC conditions described in this section) for the prepared sample and for an authentic sample of ICZ (provided by J. Bergman, Royal Institute of Technology, Stockholm, Sweden). *Caution: ICZ is structurally related to the potent toxin TCDD and should be handled with care. Contaminated labware and solutions can be routinely decontaminated with 5% sodium hypochlorite solution.*

Preparation of the Acid Reaction Mixture (RXM). Using the procedure reported by Leete and Marion (*5*), we generated the reaction mixtures at ambient temperature by treating a stirred aqueous solution of I3C (typically 0.25 mL at a concentration of

[1] Abbreviations: I3C, indole-3-carbinol; CYP, cytochrome P-450; TCDD, 2,3,7,8-tetrachlorodibenzo-*p*-dioxin; ICZ, indolo[3,2-*b*]carbazole; RXM, acid reaction mixture of I3C; 3,3'-DIM, diindol-3-ylmethane; CTr, cyclic trimer (5,6,11,12,17,18-hexahydrocyclonona[1,2-*b*:4,5-*b'*:7,8-*b''*]triindole); LTr_1, first linear trimer [[2-(indol-3-ylmethyl)indol-3-yl]indol-3-ylmethane]; LTr_2, second linear trimer [3,3-bis(indol-3-ylmethyl)-indolenine]; CTet, cyclic tetramer; LTet, linear tetramer; THF, tetrahydrofuran; DMF, dimethylformamide; *Ah* receptor, aryl hydrocarbon responsiveness receptor protein.

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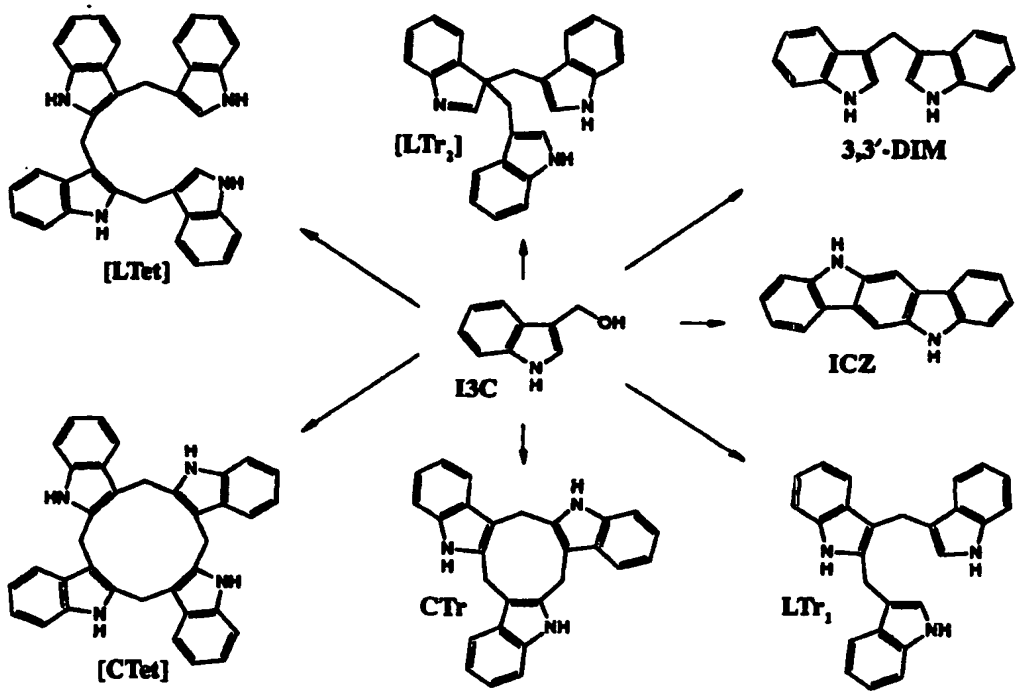
Scheme I



12 μmol/mL) with hydrochloric acid (typically 28 μL of a 1 mmol/mL solution). The solution was stirred for 10 min and then neutralized with aqueous ammonia (typically 115 μL of a 0.25 mmol/mL solution). For the HPLC analysis of the reaction products, we then added THF (typically 607 μL to give a final sample volume of 1 mL), or, for the mechanistic studies, other organic solvents and reagents, and injected portions of the solution. For preparative-scale reactions (typically starting with 50 mg of I3C in 50 mL of water), we filtered the neutralized mixture, washed the precipitate with water, and then air-dried the product while keeping it protected from ambient light.

Measurement of ICZ in RXM. For the measurement of ICZ levels in solution we prepared a calibration curve relating the fluorescence response of ICZ standard over the ranges of 3–12 ng/mL and 5–96 ng/mL. The fluorescence response was linear with respect to concentration over these ranges.

Measurement of 3,3'-DIM and Other Components in RXM. To measure 3,3'-DIM levels in the RXM solutions by HPLC, we prepared a standard solution of the authentic synthetic compound and related its peak area when detected by UV absorption at 280 nm to those in the RXM samples. For other components we assumed that the molar absorption coefficients for the various oligomers are approximately derived from that of 3,3'-DIM as follows (*5*):

$$\epsilon_{280}(\text{oligomer of order } n) = [\epsilon_{280}(3,3'\text{-DIM})][n/2]$$

where $\epsilon_{280}(3,3'\text{-DIM}) = 1.21 \times 10^4$.

Mass and NMR Spectral Data. Compound a (3,3'-DIM): *m/z* 247 (23), 246 (98, M⁺), 245 (100), 217 (19), 130 (31); δ (ppm) [CD_3CN] 4.19 (2 H, m, CH_2), 6.97 (2 H, m, ArH), 7.04 (2 H, t, *J* 1.0 Hz, H-2, H-2'), 7.09 (2 H, m, ArH), 7.35 (2 H, m, ArH), 7.51 (2 H, m, ArH), 8.99 (2 H, br s, NH).

Compound b (CTr): *m/z* 388 (31), 387 (100, M⁺), 386 (19), 372 (29), 270 (19), 269 (23), 258 (18), 257 (79), 256 (62), 255 (27), 243 (29), 130 (16); exact mass calcd for $C_{28}H_{21}N_3$: 387.1735; found: 387.1734; δ (ppm) (1) [CD_3CN, 15 °C] 3.91 (br s, CH_2 saddle), 4.01 (br d, *J* 16.5 Hz, CH_2 crown), 4.69 (br d, *J* 16.5 Hz, CH_2 crown), 6.8–7.4 (m, ArH), 7.81 (br d, ArH), 9.13 (br s, NH); (2) [CD_3CN, −5 °C], 3.92 (s, CH_2 saddle), 4.03 (d, *J* 15 Hz, CH_2 crown), 4.71 (d, *J* 15 Hz, CH_2 crown), 6.97–7.54 (m, ArH), 7.84 (d, *J* 8 Hz, ArH), 9.25 (s, NH); compare to Raverty et al. (*11*).

Compound c (LTr_1): *m/z* 376 (15), 375 (51, M⁺), 259 (24), 258 (100), 257 (91), 256 (42), 245 (17), 130 (21); exact mass calcd for $C_{26}H_{21}N_3$: 375.1735; found: 375.1729; δ (ppm) [CD_3CN] 4.26 (2 H, s, CH_2), 4.27 (2 H, s, CH_2), 6.86–6.97 (5 H, m, ArH), 7.04–7.06 (3 H, m, ArH), 7.17 (1 H, d, *J* 8 Hz, ArH), 7.28 (1 H, d, *J* 8 Hz, ArH), 7.34 (1 H, d, *J* 8 Hz, ArH), 7.36 (1 H, d, *J* 8 Hz, ArH), 7.40 (1 H, d, *J* 8 Hz, ArH), 7.50 (1 H, d, *J* 8 Hz, ArH), 8.83 (1 H, br s, NH), 8.96 (1 H, br s, NH), 9.14 (1 H, br s, NH).

Compound d (LTr_2): *m/z* 376 (14), 375 (47, M⁺), 247 (32), 246 (100), 245 (85), 244 (18), 243 (16), 217 (12), 131 (17), 130 (97), 129 (27).

Compound e (CTet): *m/z* 516 (<0.05, M⁺), 504 (1), 387 (1.6), 386 (1), 373 (2.2), 372 (3.1), 371 (3.2), 258 (100), 257 (51), 130 (7); compare to Bergman et al. (*12*).

Compound f (LTet): *m/z* 516 (1), 504 (6, M⁺), 387 (21), 375 (13), 372 (12), 259 (23), 258 (100), 257 (86), 256 (41), 246 (15), 245 (31), 243 (12), 130 (32); exact mass calcd for $C_{36}H_{32}N_6$: 504.2314; found: 504.2318.

Results

Yields and Characterization of Oligomers. The complexity of the mixture of products found in the RXM is illustrated with the HPLC chromatogram of Figure 1 where the peaks are detected using UV absorption at 280 nm. By directly measuring the molar yield for peak a (identified as 3,3'-DIM) and by computing the yields of the other components of the RXM, we estimated the corrected molar yields for each compound. By collecting the individual HPLC peaks and further purifying them through reinjection and collection, we obtained the electron impact mass spectra for each of the peaks a–f of Figure 1. We obtained sufficient amounts of the first three peaks to also record NMR spectra for these compounds. Peak a (5.9%) was identified as 3,3'-DIM by comparison of chromatographic characteristics and NMR and mass spectra to those of an authentic sample prepared according to Leete and Marion (*5*). We identified peak b (2.0%) as the symmetrical cyclic trimer 5,6,11,12,17,18-hexahydrocyclononal[1,2-*b*:4,5-*b*':7,8-*b*'']triindole (CTr) by comparison

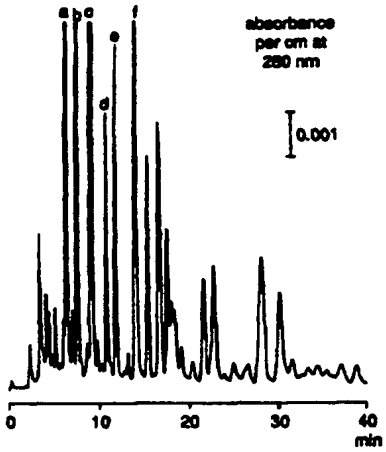


Figure 1. HPLC chromatogram of RXM [0.007-mL sample injected onto a 0.46- (i.d.) × 25-cm C-18 column using 65% acetonitrile in water as the eluting solvent] detected by UV absorption at 280 nm.

of HPLC and MS analyses with authentic material kindly provided by R. H. Thomson, Department of Chemistry, University of Aberdeen, Old Aberdeen, Scotland. NMR data also were consistent with published findings and showed a sharpening of signals in spectra run at −5 °C attributed to the bridging methylene substituents. This effect is associated with a temperature-dependent decrease in the rate of interconversion of the saddle and crown forms of the nine-membered ring of CTr. Peak c (5.9%) was identified as the linear trimer [2-(indol-3-ylmethyl)-indol-3-yl]indol-3-ylmethane (LTr_1) on the basis of a comparison of NMR and mass spectra to published data (*11*) and reasonable mechanistic arguments (see below).

The structure assignments for compounds d–f are based on their mass spectra and are ambiguous with respect to possible structural isomers involving the relative orientation of the indolyl moiety in the oligomer chains. The mass spectrum of compound d (0.6%) indicates that this compound, in contrast with LTr_1, readily fragments to form the radical cations $(C_{17}H_{12}N_2 + H)^{\cdot+}$ [m/z 246 (100)] and $C_9H_8N^{\cdot+}$ [m/z 130 (97)]. This behavior is consistent with the linear trimer 3,3-bis(indol-3-ylmethyl)indolenine (LTr_2). The mass spectral data for compounds e (0.6%) and f (1.1%), although indicating some cross contamination of the two compounds, are consistent with the assignments, respectively, of a cyclic tetramer (CTet) and a linear tetramer (LTet). The structures for these compounds, indicated in Scheme I in brackets, are proposed on the basis of analogy to the corresponding trimers and reasonable mechanistic considerations.

Kinetics and Mechanistic Studies of ICZ Formation. We identified ICZ as a component of RXM by comparison of chromatographic, fluorescence emission, ultraviolet absorption, and HPLC–mass spectral characteristics with those of an authentic sample as described in detail elsewhere (*7*). We measured the levels of ICZ present in the reaction mixtures using HPLC analysis with fluorescence detection (Table I). ICZ is only slowly formed in a purely aqueous environment with molar yield after 48 h of 20 ppm. However, after adding THF or acetonitrile to the reaction emulsion to produce a homogeneous solution, we observed a molar yield after 48 h of 91 ppm.

We investigated several mechanistic possibilities for ICZ formation by modifying the initial reaction conditions. In one experiment we examined the possible involvement of

Table I. ICZ Concentrations Measured in Various Samples

sample	sample size, mL	ICZ found, pmol	molar, ppm[a]
RXM at 10 min (aqueous)	0.25	5.9	2.0
RXM at 20 h (aqueous)	0.25	20	6.6
RXM at 48 h (aqueous)	0.25	60	20
RXM at 20 h (organic/aq)[b]	1.0	220	74
RXM at 48 h (organic/aq)	1.0	270	91

[a] Based on starting amount or dose of I3C. [b] 393 μL of water (pH 7–8) plus 607 μL of THF.

Table II. pH Dependence of Trimers Formed in RXM

pH	average trimers:3,3′-DIM ratio[a]
0	2.4
1	1.6
2	0.9
3	0.7

[a] Sum of peak areas for linear and cyclic trimer divided by the peak area for 3,3′-DIM (starting I3C concentration 12 μmol/mL).

radical processes. We attempted to exclude oxygen during and after the acid-treatment period by sparging all solutions with helium and keeping the reaction samples under a nitrogen atmosphere before and during analysis. We found that the exclusion of oxygen increased ICZ yield at 15 min 2.8-fold, but that by 24 h ICZ yield was only 1.3-fold greater than in control samples run with exposure to ambient oxygen. During the post-acid-treatment period, addition of benzophenone at 2.8 μmol/mL and air to the THF solution caused no significant difference in the amount of ICZ formed at 15 min (0.995-fold less formed) compared to control conditions. By changing the solvent added to the neutralized solution from THF to the more polar dimethylformamide (DMF), we observed that ICZ was formed about 2 times as fast [0.78 vs an average rate of 0.38 pmol/(mL·min)]. Addition of the radical scavenger *p*-benzoquinone at 3 μmol/mL in THF to the neutralized solution did not produce a significant change in the rate of ICZ formation [measured as 0.18 pmol/(mL·min)] compared to the rate for THF alone [which varied from 0.23 to 0.52 pmol/(mL·min)].

In another experiment we tested for possible roles of 3,3′-DIM or formaldehyde in the formation of ICZ and other oligomers. We treated 3,3′-DIM stirred as a slurry in water to the same acid conditions used to generate the RXM solution. Following the usual addition of aqueous ammonia and THF, we submitted the sample to HPLC analysis. We found no peaks other than those present in a control sample that had not been treated with the hydrochloric acid. This result indicates that 3,3′-DIM is stable to the reaction conditions and suggests that 3,3′-DIM is not an intermediate by itself in the oligomerization of I3C. Similarly, addition of formaldehyde (6 mmol/mL) to the standard acidic reaction conditions resulted in no significant change in the rate of ICZ formation compared to the standard procedure. This result suggests that free formaldehyde formed by cleavage of 3-(hydroxymethyl)-indoles is not a likely participant in the oligomerization process.

Effect of pH on Oligomerization. We measured the effect of pH on the rate of ICZ formation and on the distribution of products in the RXM. The ratios of peak areas as measured by HPLC using UV detection for LTr_1 and CTr relative to 3,3′-DIM are summarized in Table II. The trend is for formation of more of the trimers relative to 3,3′-DIM as the pH decreases. The effect of pH on the initial ICZ level after 10 min of acidic treatment is shown in Figure 2. A critical region around pH 1.5 and above

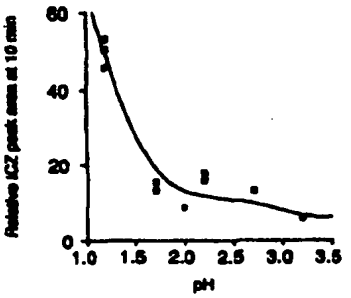


Figure 2. pH dependence of the amount of ICZ formed after 10 min in the aqueous-acid treatment of I3C. The HPLC peak areas for ICZ at 10 min are derived from the interpolation of the graph of peak areas vs time for 3–5 time points per sample. The HPLC chromatograms measured fluorescence emission at 405 nm with excitation at 335 nm using the conditions described in the Experimental Section. The smoothing curve is fitted by distance-weighted least squares using SYSTAT 5.1 for the Macintosh.

Table III. Concentration Dependence of Trimers Formed in RXM

I3C concn, μmol/mL	dilution ratio	average trimers:3,3′-DIM ratio[a]
1.2	0.1	5.5
2.4	0.2	3.8
6.0	0.5	2.1
12	1.0	1.6

[a] Sum of peak areas for linear and cyclic trimer divided by the peak area for 3,3′-DIM (reaction at pH 1).

results in significantly reduced levels of ICZ formed.

Dilution Effect on the Product Distribution. We also measured the effect of the initial concentration of I3C on the approximate distribution of LTr_1 and CTr formed relative to 3,3′-DIM. The results (Table III) indicate a trend toward relatively more trimers as the initial concentration of I3C is decreased. This result is as expected since increased time in solution is expected to facilitate formation of higher order oligomers and polymers.

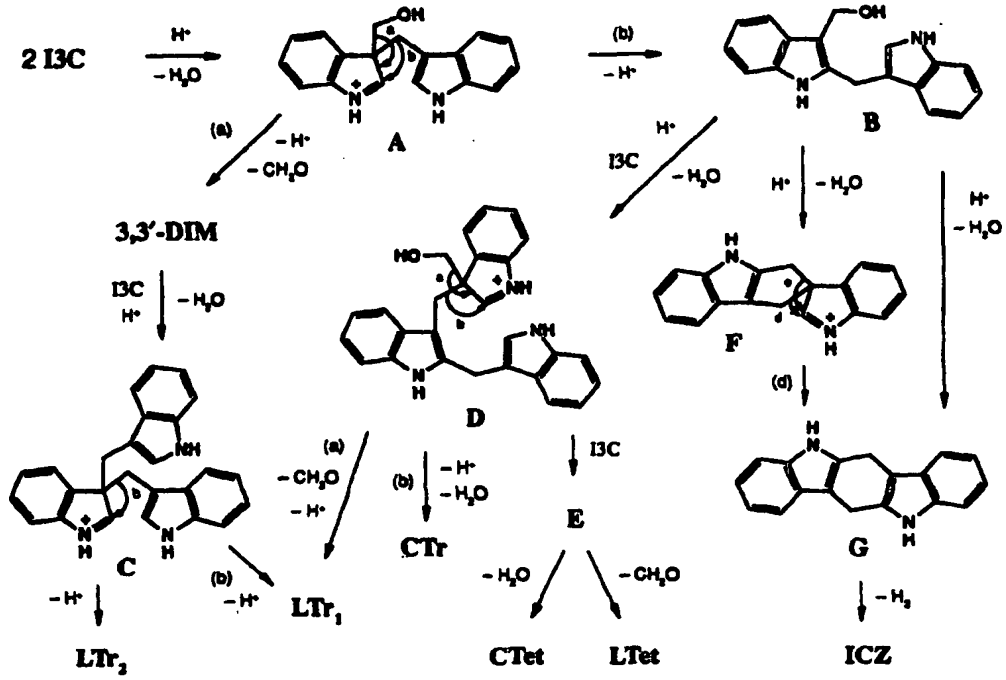
Discussion

Although the product composition observed in the present studies is complex, the identities of the major indolic products are generally in accord with expectation based on published studies of indole oligomerization under various acidic and thermal conditions. Amat-Guerri et al. (*13*) isolated 3,3′-DIM and LTr_1 following treatment of I3C with mild acid (pH 5) in aqueous methanol. Raverty and Thomson (*11*) identified 3,3′-DIM and trimers LTr_1 and CTr as products of the high-temperature treatment of diethyl(indol-3-yl)ammonium chloride in dimethyl sulfoxide solution. Treatment of indoles with formaldehyde under strongly acidic conditions in methanol was shown by Bergman et al. (*12*) to produce a variety of products. These included a cyclic tetramer which was suggested to have the structure of the symmetrical product CTet. Evidence for the production of LTr_2 or LTet has not been presented. Processes employing methanolic solutions (and thus homogeneous reaction conditions) generally can produce each oligomer in higher yield (15–90%) than do processes employing thermal and purely aqueous conditions (less than 6%). This effect of methanol is probably due to increased solubility of starting materials and of virtually all the intermediates produced during the reaction.

Production of ICZ under purely aqueous conditions has not been reported. However, ICZ is produced in approximately 20% yield by reaction in strongly acidic methanol solutions of indole and formaldehyde or of 3,3′-DIM by itself, if the reactions are run in the presence of light, oxygen, and photosensitizer (*14*). Indoles are highly reactive under these photodynamic conditions (*15*). In addition, Rannug et al. have reported the conversion of tryptophan under photodynamic conditions to unidentified products with high affinity for the *Ah* receptor (*16*). However, the importance of these conditions in the present studies is questionable because they are not likely to occur in the gastrointestinal tract. We therefore sought to determine whether ICZ production in our aqueous system could be attributed to a photodynamic effect. Our observation that exclusion of oxygen during and after the acid treatment resulted in an initial increase in rate of ICZ formation is consistent with nonradical mechanisms during both phases of the reaction. Our observation of no change in the rate of ICZ production following addition to the neutralized, aerated solution of either the photosensitizer benzophenone or the radical scavenger *p*-benzoquinone also indicates that ICZ formation in our neutralized reaction mixture does not involve a radical process. Evidence that a heterolytic process occurs in the period after acid treatment is the near doubling in the rate of ICZ formation on changing of solvent from THF to the more polar DMF (*17*). Additional evidence that the reactions run under methanolic and aqueous conditions proceed by different pathways is that, in contrast with the reaction run in acidic methanol, 3,3′-DIM is stable to our aqueous acidic conditions and addition of formaldehyde to our standard conditions did not affect ICZ yield. Taken together, our results support the notion that a precursor of ICZ, possibly dihydroindolocarbazole (G, Scheme II), is formed in aqueous acidic solution and aromatized by a heterolytic dehydrogenation process under conditions that can occur in vivo.

Our results on the effects of pH on the oligomerization process are an extension of the findings of Amat-Guerri et al. (*15*) and provide evidence for a mechanistic rationale for the observed product distribution. Under neutral conditions no polymerization beyond the dimer is observed, and under weakly acidic conditions (pH 5) dimer and LTr_1 are formed almost exclusively (*15*). As the pH is lowered further, yields of ICZ and larger oligomers are increased relative to that of 3,3′-DIM. A mechanistic rationale for these observations is provided in Scheme II. The expected facile acid-catalyzed dehydration of the vinylogous hemiaminal, I3C, provides 3-methyleneindolenininium cation as suggested originally by Leete and Marion (*5*). Nucleophilic attack by another I3C molecule provides the intermediate A which can either expel formaldehyde (process a) to form 3,3′-DIM or undergo a 1–2 sigmatropic shift (process b) to form compound B. The indolenininium ion A (equivalent to 3*H*-indolium ion) has been proposed previously as an intermediate in the enzymatic conversion of indole-3-acetic acid to 3,3′-DIM (*18*). Dimeric 2-substituted indoles analogous to B have been isolated as products of reactions of 3-substituted indoles under enzymatic (*19*) and nonenzymatic (*13*) conditions. The observation of Amat-Guerri et al. (*13*) that the oligomerization process goes only as far as dimer under neutral conditions indicates that the unprotonated form of A reacts preferably by pathway a. Process b is increasingly preferred as the pH of the reaction decreases which, in addition to the fact that 3,3′-DIM is stable in aqueous acid, accounts for the observed decrease in yields of 3,3′-DIM relative to other products as the acidity of the reaction mixture is increased.

Scheme II



Although the composition of oligomers is complex, only a single orientation of the indoles is present in each of the major oligomers and ICZ. No evidence was obtained by us or other investigators for multiple cyclic trimers or for more than one indolocarbazole. This comparative simplicity of major products is explained on the basis of established susceptibility of indoles to electrophilic attack at the C-2 and C-3 positions (*20*, *21*). Thus, the likely intermediate dihydroindolocarbazole G can be produced either directly by intramolecular attack at C-2′ of intermediate B or indirectly, as proposed by Bergman (*14*), by attack at C-3′ to produce intermediate F followed by a sigmatropic shift (process d). Spiropentanes of this type have been isolated as products of benzcarbazole synthesis from 3-substituted indoles (*21*). The alternative shift (process a) of intermediate F is less preferred than process d because of the expected decreased migratory aptitude of the C-2 versus the C-3 methyleneindolium ion in which the nitrogen atom lone pair participates in the resonance stabilization of the positive charge without disrupting the benzene ring aromaticity. In like manner, intermolecular reaction of B with I3C produces intermediate D which can (1) lose formaldehyde (process a) to give LTr_1, (2) undergo shift b followed by cyclization to give CTr, or (3) react with I3C to produce tetramers by analogous processes. Formation of LTr_2 can be rationalized by an electrophilic attack of 3-methyleneindoleninium ion on 3,3′-DIM to produce intermediate C, which can rearrange by process b to form LTr_1 or deprotonate to form LTr_2.

These studies confirm that while product yields may vary greatly, the oligomeric products of 3-substituted indoles produced under diverse conditions are remarkably similar. Thus, treatment of certain 3-substituted indoles under enzymatic, photodynamic, thermal, or the range of acidic conditions referred to in this report can produce similar products. It appears likely that biologically active substances analogous in structure to 3-methyleneindole oligomers or ICZ may be produced from other commonly occurring 3-substituted indoles such as tryptophan and skatole.

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Flavin-Containing Monooxygenase-Dependent Stereoselective S-Oxygenation and Cytotoxicity of Cysteine S-Conjugates and Mercapturates

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The metabolism of cysteine S-conjugates of both *cis*- and *trans*-1,3-dichloropropene in the presence of rat kidney microsomes and purified flavin-containing monooxygenase from hog liver was investigated in vitro. Preliminary studies with isolated rat kidney cells demonstrated that cysteine S-conjugates were quite toxic to the cells in a process which was consistent with a role of the flavin-containing monooxygenase in the bioactivation of the nephrotoxins. Putative *S*-oxide metabolites of cysteine S-conjugates were chemically synthesized, and diastereomers were separated and identified by spectroscopic means. The metabolic products of cysteine S-conjugates were identified by comparing the chemical properties of the metabolites with authentic synthetic cysteine S-conjugate *S*-oxides. Surprisingly, S-conjugate *S*-oxygenase activity was not observed with rat kidney microsomes but was present when cysteine S-conjugates were incubated with the highly purified flavin-containing monooxygenase from hog liver. The kinetic parameters indicated that considerable *S*-oxygenase stereoselectivity and structural selectivity was observed: cis cysteine S-conjugates were preferred substrates and N-acetylation of cysteine S-conjugates decreased substrate activity. S-Oxygenation was considerably diastereoselective and diastereoselectivity was much greater for cysteine S-conjugates with higher V_{max} values. Cysteine S-conjugate *S*-oxides were not indefinitely stable, and under certain conditions, the *S*-oxides underwent a [2,3]-sigmatropic rearrangement to acrolein. Formation of acrolein or other electrophilic products from *S*-(chloropropenyl)cysteine conjugate *S*-oxides may contribute to the renal effects observed for *S*-(chloropropenyl)cysteine conjugates. Thus, cytotoxicity studies with isolated rat proximal tubular cells or LLC-PK1 cells treated with cysteine S-conjugates showed a time- and dose-dependent decrease in cell viability. Reduction of renal cytotoxicity of cysteine S-conjugates in the presence of methimazole, an alternate substrate competitive inhibitor of the flavin-containing monooxygenase, suggested that this enzyme may contribute to the renal effects of 1,3-dichloropropene.

Introduction

Although glutathione S-conjugate formation is an important detoxication process, reports of haloalkane and haloalkene bioactivation via glutathione S-conjugate formation have appeared (for reviews, see refs 1 and 2). The glutathione-dependent bioactivation of vicinal dihaloalkanes involves the intermediate formation of half-sulfur mustards that give rise to reactive episulfonium ions, which are mutagenic and nephrotoxic (*3*). A range of nephrotoxic haloalkenes are bioactivated via glutathione S-conjugate formation, metabolism of the glutathione S-conjugates to cysteine S-conjugates, translocation to the kidney, and renal bioactivation by cysteine conjugate β-lyase (*4*).

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Biochemistry

Aromatic hydrocarbon responsiveness-receptor agonists generated from indole-3-carbinol *in vitro* and *in vivo*: Comparisons with 2,3,7,8-tetrachlorodibenzo-*p*-dioxin

(anticarcinogen/*Brassica oleracea*/indolo[3,2-*b*]carbazole)

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ABSTRACT Indole-3-carbinol (I3C) is a secondary plant metabolite produced in vegetables of the *Brassica* genus, including cabbage, cauliflower, and brussels sprouts. I3C is both an anti-initiator and a promoter of carcinogenesis. Consumption of I3C by humans and rodents can lead to marked increases in activities of cytochrome P-450-dependent monooxygenases and in a variety of phase II drug-metabolizing enzymes. We have reported previously that the enzyme-inducing activity of I3C is mediated through a mechanism requiring exposure of the compound to the low-pH environment of the stomach. We report here the aromatic hydrocarbon responsiveness-receptor K_d values (22 nM–90 nM), determined with C57BL/6J mouse liver cytosol and the *in vitro*- and *in vivo*-molar yields (0.1–6%) of the major acid condensation products of I3C. We also show that indolo[3,2-*b*]carbazole (ICZ) is produced from I3C in yields on the order of 0.01% *in vitro* and, after oral intubation, *in vivo*. ICZ has a K_d of 190 pM for aromatic hydrocarbon responsiveness-receptor binding and an EC_{50} of 269 nM for induction of cytochrome P4501A1, as measured by ethoxyresorufin O-deethylase activity in murine hepatoma Hepa 1c1c7 cells. The binding affinity of ICZ is only a factor of 3.7×10^{-2} lower than that of the highly toxic environmental contaminant and cancer promoter 2,3,7,8-tetrachlorodibenzo-*p*-dioxin. ICZ and related condensation products appear responsible for the enzyme-inducing effects of dietary I3C.

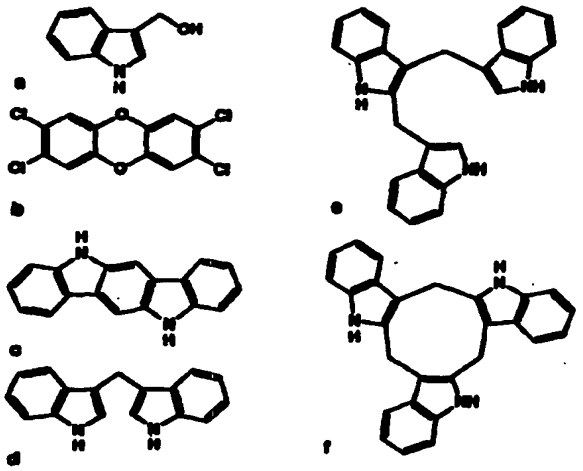

FIG. 1. (Compound a) I3C. (Analog b) 2,3,7,8-Tetrachlorodibenzo-*p*-dioxin (TCDD). (Analog c) Indolo[3,2-*b*]carbazole (ICZ). (Analog d) 3,3′-Diindolylmethane. (Analog e) 2-(Indol-3-ylmethyl)-3,3′-diindolylmethane (LT). (Analog f) 5,6,11,12,17,18-Hexahydrocyclonona[1,2-*b*:4,5-*b*′:7,8-*b*″]triindole (CT).

Indole-3-carbinol (I3C) (Fig. 1 compound a) is an autolysis product of glucobrassicin (3-indolylmethyl glucosinolate), a compound that occurs naturally in large amounts in a number of vegetables of the *Brassica* genus (e.g., cabbage, 0.1–1.9 mmol/kg of fresh weight; cauliflower, 0.1–1.6 mmol/kg; and brussels sprouts, 0.5–3.2 mmol/kg) (1–3). I3C has received considerable attention as a dietary modulator of carcinogenesis (4). When administered before carcinogen exposure, I3C reduces both the incidence of neoplasia and the formation of covalent adducts of carcinogen with DNA (5–7). When administered after carcinogen exposure, I3C increases neoplastic outcome and, thus, promotes carcinogenesis (8–10).

The mechanism(s) by which I3C modulates carcinogenesis may be related to its potency as an inducer of enzymes involved in the metabolism of carcinogens and other foreign chemicals (5). In rodent models, oral administration of low levels of I3C significantly increased activities of epoxide hydrolases, quinone reductase, and cytochrome P4501A1 (CYP1A1¶)-dependent monooxygenase, and at high levels increases in glutathione S-transferases were observed (5, 12, 13). In humans, the effects of I3C appear similar. Human volunteers exposed to purified I3C showed increases in the activity of estradiol 2-hydroxylase (14). Human volunteers consuming *Brassica* vegetables also showed marked increases in the oxidative metabolism of phenacetin and antipyrine (15), as well as in the glucuronidation of acetaminophen (16).

We have been interested in the mechanism by which I3C induces CYP1A1-dependent monooxygenase activity and in its properties as a modulator of carcinogenesis. Several experimental observations have provided support for the idea that I3C is activated via an acid-catalyzed reaction occurring in the low-pH environment of the stomach. Evidence to support this hypothesis includes the observations that (*i*) oral, but not i.p., administration of I3C led to an induction of hepatic CYP1A-dependent monooxygenase activities (12, 17); (*ii*) acid treatment of I3C generated a reaction mixture that induced monooxygenase activity after i.p. and oral administration (12); and (*iii*) results of structure–activity

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Abbreviations: I3C, indole-3-carbinol; ICZ, indolo[3,2-*b*]carbazole; LT (linear trimer), 2-(indol-3-ylmethyl)-3,3′-diindolylmethane; CT (cyclic trimer), 5,6,11,12,17,18-hexahydrocyclonona[1,2-*b*:4,5-*b*′:7,8-*b*″]triindole; TCDD, 2,3,7,8-tetrachlorodibenzo-*p*-dioxin; TCDF, 2,3,7,8-tetrachlorodibenzofuran; Ah, aromatic hydrocarbon responsiveness.

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¶Cytochrome P-450 nomenclature is according to Nebert *et al.* (11).

studies indicated that indoles unstable at acidic pH have greater potency as inducers of monooxygenase activity than do indoles stable at acidic pH (12). In this report, we present results of experiments designed to further describe the mechanism(s) of action of I3C. First, we show that I3C is converted in potentially significant yields *in vivo* and *in vitro* to ICZ and other methyleneindole condensation products (Fig. 1). Second, we report the aromatic hydrocarbon responsiveness (Ah)-receptor-binding characteristics of these indole derivatives, and third, we characterize the potency of ICZ as an inducer of CYP1A1 activity in murine hepatoma cells, Hepa 1c1c7.

MATERIALS AND METHODS

Chemicals. We purchased indole-3-carboxaldehyde, indole-3-acetonitrile, and I3C from Aldrich. These indoles were recrystallized immediately before use. We prepared and recrystallized 3,3'-diindolylmethane (Fig. 1 analog d) and ICZ (Fig. 1 analog c) according to published methods (18, 19). We isolated 2-(indol-3-ylmethyl)-3,3'-diindolylmethane (LT) (Fig. 1 analog e) and 5,6,11,12,17,18-hexahydrocyclonona[1,2-*b*:4,5-*b'*:7,8-*b''*]triindole] (CT) (Fig. 1 analog f) from the acid reaction mixture of I3C by HPLC using conditions described below. Purities of trimer samples were established by HPLC analyses as >98%. Identities were established by comparing NMR and mass spectra to published data (20, 21). TCDD (Fig. 1 analog b) was a gift from B. N. Ames (University of California, Berkeley, CA). 2,3,7,8-Tetrachlorodibenzofuran (TCDF) and radioligand [2-^{125}I]7,8-dibromodibenzo-*p*-dioxin were gifts from A. Poland (McArdle Laboratory for Cancer Research, Madison, WI). We purchased resorufin from Aldrich. We prepared and purified ethoxyresorufin according to published procedures (22).

Analysis of Acid Condensation Products of I3C *in Vitro* and *in Vivo*. An acid reaction mixture was generated at ambient temperature by treating a stirred aqueous solution of I3C (1 mg/ml) with 1 M hydrochloric acid. The reaction was neutralized with 0.25 M aqueous ammonia, diluted with tetrahydrofuran, 60:40, and analyzed by HPLC using a C_{18} bonded-phase column with acetonitrile in water as the mobile phase. To monitor production of most condensation products, we used UV absorption at 280 nm and calibration against known standards. We used a fluorescence detector for routine analyses of ICZ. HPLC-MS (Hewlett–Packard model HP5988) was used to confirm peak identity for ICZ and 3,3'-diindolylmethane.

To monitor production of condensation products from I3C in the gastrointestinal tract, we dosed male Sprague–Dawley rats by oral intubation with I3C (73.5 mg/kg of body weight) in corn oil, euthanized them, and then excised their gastrointestinal tracts and contents. The ethyl acetate extracts of sucrose/phosphate (pH 7.4) homogenates were then filtered through a nylon membrane and analyzed by HPLC. I3C and the acid reaction mixture products are stable under these extraction conditions. Thus, products identified *in vivo* are not produced as artifacts of the analytical procedures.

Characterization of I3C Condensation Products as Agonists of the Ah Receptor. To determine affinity of compounds for the Ah receptor, we conducted competitive binding experiments with the radioligand [2-^{125}I]7,8-dibromodibenzo-*p*-dioxin. Bound and free radioligand were separated by the charcoal-adsorption assay with Ah receptor prepared from C57BL/6J mouse liver cytosol (23).

Analysis of Induction of CYP1A1-Dependent Activity. To compare the capacity of a purified compound or reaction mixture to induce CYP1A1 activity, we monitored the induction of ethoxyresorufin *O*-deethylase in cultured murine hepatoma cells (24). Results of previous experiments have shown that I3C treatment increases the levels of *CYP1A1* gene transcripts in the cytosol (25). Briefly, confluent Hepa 1c1c7 cells were treated with different concentrations of the test materials dissolved in dimethyl sulfoxide (final dimethyl sulfoxide concentration of 1.5% (vol/vol) in the growth medium). After 2 days of incubation at 37°C, 3×10^6 cells were suspended in ≈1.5 ml of isotonic NaCl/phosphate buffer at pH 7.4 in a 1-cm-square fluorometer cell. To the cell suspension we then added 0.5 ml of a solution of ethoxyresorufin in the same buffer to make the final ethoxyresorufin concentration 2.5 μM in the cuvette. To monitor resorufin production, we recorded the fluorescence produced at 586 nm from excitation at 510 nm. We calibrated the fluorescence measurements with authentic resorufin.

RESULTS AND DISCUSSION

HPLC analysis indicated that within 10 min in aqueous acid I3C is converted to a complex mixture. The three most prevalent UV-absorbing compounds in the mixture were LT, CT, and 3,3'-diindolylmethane (Fig. 1). Molar yields of these compounds were in the range of 2–6% of original I3C. Production of 3,3'-diindolylmethane and LT by acid treatment of I3C is consistent with previous findings (20). Further analysis of the acid reaction mixture of I3C using HPLC with fluorescence detector indicated ICZ (Fig. 1). This discovery is of particular interest because ICZ is nearly isosteric with TCDD and binds with high affinity to the Ah receptor (26). Thus, ICZ was probably a potent inducer of CYP1A1 (27–30). The evidence that ICZ is a component of the acid reaction mixture of I3C is as follows: (*i*) no ICZ peak was detectable by HPLC analysis of purified I3C; (*ii*) the presumed ICZ peak in the chromatogram of the acid reaction mixture had the same retention time and peak shape as did authentic ICZ under a range of HPLC elution conditions (Fig. 2); (*iii*) chromatographic analysis showed that spiking of the reaction mixture with authentic ICZ appropriately increased the area of the presumed ICZ peak, and no additional peaks were produced (data not shown); (*iv*) the fluorescence emission and excitation spectra of the presumed ICZ peak of the reaction mixture were identical to those of the authentic compound (Fig. 3); and finally, (*v*) HPLC-MS analyses for *m/z* 256 and 128, the two major ions present in the electron-

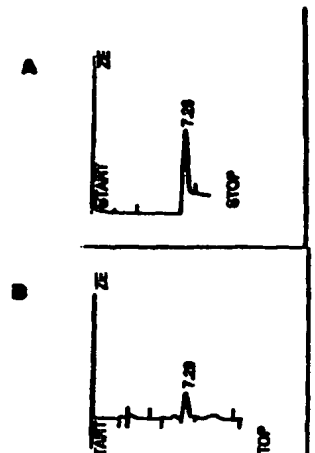

FIG. 2. (*A*) Chromatogram of ICZ (C_{18} stationary phase with 65% acetonitrile in water as eluting solvent) detected with fluorescence excitation at 335 nm and emission at 405 nm. (*B*) Chromatogram of the acid reaction mixture of I3C with fluorescence detection.

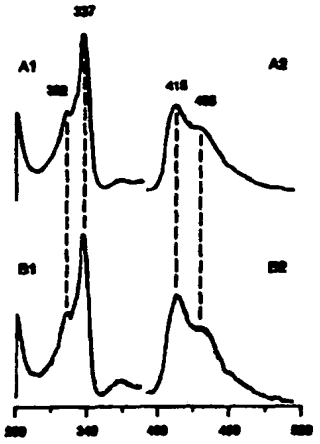

FIG. 3. Fluorescence excitation and emission spectra for the presumed ICZ peak (*A1* and *A2*, respectively) from the acid reaction mixture of I3C and from authentic ICZ (*B1* and *B2*, respectively). Spectra were obtained by scanning the HPLC peaks stopped in the detector flow cell. The fluorescence emission was measured at 415 nm in *A1* and *B1*, and the excitation was 335 nm in *A2* and *B2*.

impact mass spectrum of the authentic ICZ, indicated these masses in the proper ratio in the mass spectrum of the presumed ICZ peak (Fig. 4).

Quantitative analysis by HPLC indicated that *in vitro* yields of ICZ increased slowly with time. After 10 min of treatment with acid, the ICZ molar yield was 0.0002%; after 48 hr, the ICZ yield was 0.002%. However, when the acid-treatment mixture produced after 10 min was neutralized and diluted with tetrahydrofuran, ICZ yields were as high as 0.0075% at 20 hr and 0.0090% at 48 hr.

To determine whether the acid-condensation products are generated *in vivo*, we analyzed gastric and intestinal contents of rats after oral administration of I3C. Five hours after oral gavage with I3C, 3,3'-diindolylmethane, LT, and CT were readily detected in the small intestine (data not shown). Molar yields were ~0.1–0.34% from I3C. ICZ was present in molar yields of 0.0016% in stomach tissue and contents of stomach and 0.0011% in contents of small intestine (Fig. 5). Twenty hours after I3C treatment, the yield of ICZ in stomach tissue and contents was only 0.0001%, and much higher yields were found in the contents of cecum (0.0088%) and in feces (0.0010%). These 20-hr figures suggest a minimum total *in vivo* yield for ICZ on the order of 0.01%. None of these values is corrected for recovery and, therefore, they reflect minimum levels of ICZ and other condensation products in the samples. The identities of the ICZ and 3,3'-diindolylmethane produced *in vivo* were confirmed by comparison of their HPLC retention times, fluorescence or UV spectra, and HPLC-MS behaviors with those of the authentic compounds.

To begin to evaluate the biological activities of I3C acid products, we compared the Ah receptor-binding affinities of the more prevalent condensation products to those of I3C and related indole monomers (Table 1, Fig. 6). In these experiments, TCDD or TCDF, two of the most potent Ah-receptor agonists known, were used as positive controls. Our results were consistent with previous findings (26, 27) but indicated that ICZ binds to the Ah receptor with K_d of 190 pM, which is similar but clearly different from the K_d for TCDD (7.1 pM). This result indicates that the receptor-binding affinity for ICZ is only 3.7×10^{-2}- and 8.4×10^{-2}-fold lower than those of TCDD and TCDF, respectively. LT, CT, and 3,3'-diindolylmethane bind with considerably less affinity and had K_d values of 22 nM, 62 nM, and 90 nM, respectively. These

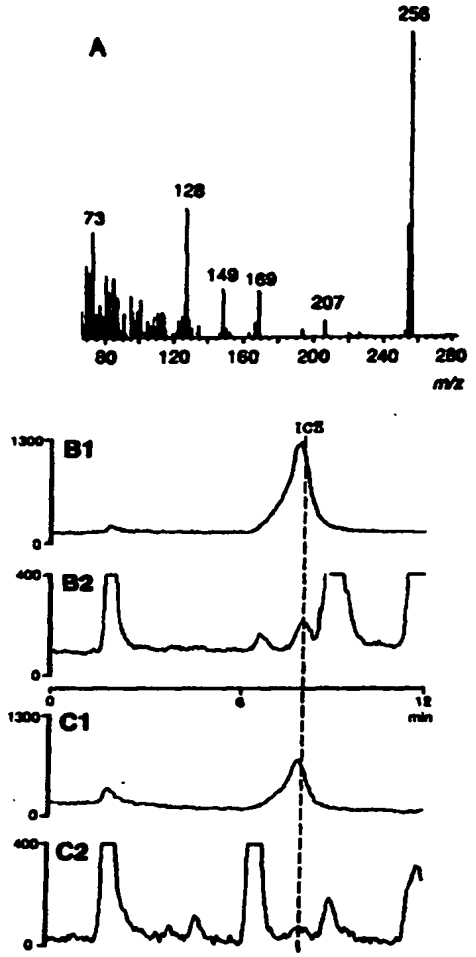

FIG. 4. (*A*) Electron impact mass spectrum of ICZ standard. (*B1*) Chromatogram of ICZ (C_{18} stationary phase with 60% acetonitrile in water as eluting solvent) detected using electron impact single-ion monitoring at *m/z* 256. (*B2*) Chromatogram of the acid reaction mixture of I3C detected with single-ion monitoring at *m/z* 256. (*C1*) Chromatogram of ICZ detected with single-ion monitoring at *m/z* 128. (*C2*) Chromatogram of the acid reaction mixture of I3C detected with single-ion monitoring at *m/z* 128. Vertical axes in *B* and *C* chromatograms represent adjusted ion counts measured for the monitored *m/z* values.

receptor-binding affinities are, thus, between 3×10^{-4}- and 8×10^{-5}-fold lower than that of TCDD.

We found that the simple 3-substituted indoles I3C, indole-3-carboxaldehyde, and indole-3-acetonitrile bound to the Ah receptor very weakly, if at all. Given the high concentrations of these indoles required to displace the radioligand, it seems probable that the observed competition may result from a trace contaminant in the indole preparation or from the generation of condensation products in solution during the 18-hr incubation time. In support of our contention that I3C is not an Ah-receptor agonist, we observed that, of all indoles tested, I3C has the lowest binding affinity for the Ah receptor. The relative binding affinities of these indoles sharply contrast with the biological potency of these congeners as inducers of monooxygenase activity in the whole animal, where I3C is the most potent inducer (12). These observations indicated that none of the simple 3-substituted indoles, including I3C, is an agonist of the Ah receptor *in vivo*.

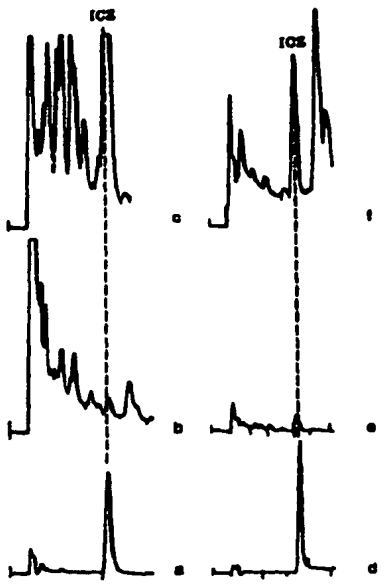

FIG. 5. HPLC (column, C_{18} stationary phase with 65% acetonitrile in 31 mmol/liter of ammonium phosphate buffer as eluting solvent; detector, fluorescence emission at 405 nm and excitation at 335 nm) chromatograms of ICZ (*a*) (detector scale at 4×); cecal contents of control rat in 20-hr group (*b*) (sample undiluted); cecal contents of I3C-treated rat in 20-hr group (*c*) (sample diluted 1:10); ICZ (*d*) (detector scale at 1×); stomach contents of control rat in 5-hr group (*e*) (sample diluted 1:10); and stomach contents of I3C-treated rat in 5-hr group (*f*) (sample diluted 1:10).

Our results from ethoxyresorufin *O*-deethylase (EROD) induction experiments in Hepa 1c1c7 cells indicated that the acid reaction mixture produced maximum induction at 100 μM (I3C equivalents), and above this dose it was highly toxic to the cells. The EC_{50} value for the reaction mixture was at least 70 μM. By contrast, the EC_{50} values for ICZ and TCDD were considerably less at 260 ± 50 nM and 36 ± 12 pM, respectively (Fig. 7). Neither ICZ nor TCDD showed signs of toxicity even at the higher doses where monooxygenase activity declined. Our value for the EC_{50} for TCDD in Hepa 1c1c7 cells is similar to the value reported by Israel and Whitlock (33).

The characteristics of these substances, as agonists of the Ah receptor, may be summarized as follows: simple 3-substituted indoles, such as I3C, do not bind to the Ah receptor with high affinity and may not be significant agonists of the Ah receptor *in vivo*. Upon contact with acid, both *in vitro* and *in vivo*, a series of indole condensation products is generated

Table 1. Binding affinities for the Ah receptor

Compound	K_d, M	Relative binding affinity
TCDD	7.1×10^{-12}	1.00
TCDF	1.6×10^{-11}	0.44
ICZ	1.9×10^{-10}	3.7×10^{-2}
LT	2.2×10^{-8}	3.3×10^{-4}
CT	6.2×10^{-8}	1.1×10^{-4}
3,3'-Diindolylmethane	9.0×10^{-8}	7.8×10^{-5}
Indole-3-carboxaldehyde	5.1×10^{-6}	1.4×10^{-6}
Indole-3-acetonitrile	7.6×10^{-6}	9.3×10^{-7}
I3C	2.7×10^{-5}	2.6×10^{-7}

The competitive binding assay was done as described (31). The true K_d of the radioligand was assumed to be 6.5×10^{-12} (31); the K_d for each compound was determined by the method of Linden (32).

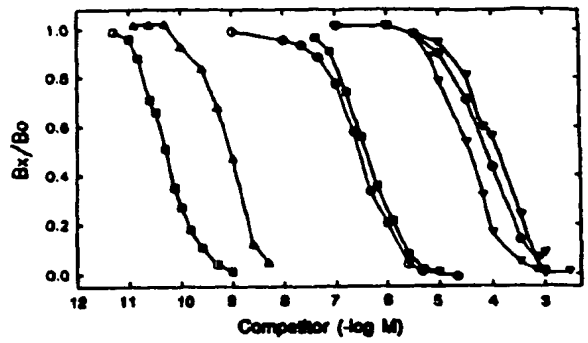

FIG. 6. Competition of TCDD and I3C condensation products for the specific binding of [2-^{125}I] 7,8-dibromodibenzo-*p*-dioxin to the Ah receptor. The competition-binding assay was done as described. Assay conditions were as follows: total radioligand concentration of 5–10 pM, total receptor concentration of 20 pM, and various concentrations of competitor (abscissa). Specific binding was determined on 1-ml reaction volumes after an 18-hr incubation at 4°C. Ordinate is B_x/B_o, specifically bound radioligand in the presence of a given amount of competitor (B_x) divided by specifically bound radioligand in the absence of competitor (B_o). Compounds were dissolved in dimethyl sulfoxide and added to the incubation in 5-μl volumes. Each data point represents the average value from at least two determinations. □, TCDD; △, ICZ; ○, CT; ■, 3,3'-diindolylmethane; ▽, indole-3-carboxaldehyde; ●, indole-3-acetonitrile; ▽, I3C. Curves for TCDF and LT are similar in shape and closely overlap curves for TCDD and CT, respectively, and were omitted for clarity.

from I3C. This reaction mixture is composed primarily of indole condensation products, which apparently bind weakly to the Ah receptor. However, whether this weak binding activity is a property of the major oligomers themselves or is due to small amounts of ICZ produced during the assay procedure is not resolved by the present studies. By far, the most potent Ah receptor agonist identified in the reaction mixture is ICZ. Because of the higher yields of the weaker binding oligomers, ICZ appears of roughly equal importance to the other oligomers in the *in vivo* enzyme-inducing activity of the mixture. Comparisons of ICZ and TCDD indicate that both have high affinities for the Ah-receptor protein, have low toxicities for the Hepa 1c1c7 cells, and are potent inducers of CYP1A1 in cell culture. The difference we observe in inducing EC_{50} values for ICZ and TCDD from EROD assay (≈7000 fold) is larger than would be predicted

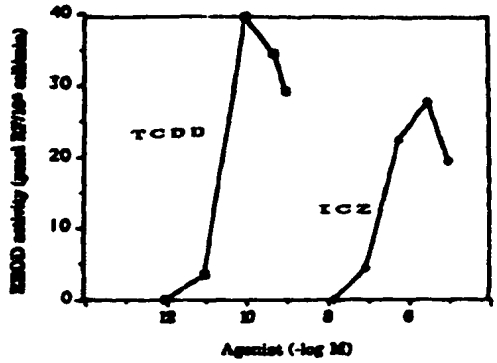

FIG. 7. Ethoxyresorufin *O*-deethylase activity (EROD) was determined in Hepa 1c1c7 cells as described. Computed mean EC_{50} values are 36 ± 12 pM and 260 ± 50 nM for TCDD and ICZ, respectively. (EC_{50} values are means ± SDs of three determinations for TCDD and five determinations for ICZ.)

from the observed differences in *in vitro* binding affinities (3.7 × 10^{-2} fold) and may arise from, among other causes, variations in cellular uptake, metabolic rate, or energy-dependent inducer efflux associated with multidrug resistance for the two compounds (34).

Because the Ah-receptor-binding and the CYP1A1-inducing characteristics of ICZ and TCDD are similar, their biological effects may be similar in other respects also. Indeed, TCDD and ICZ are both active in reducing lymphoid development in murine fetal thymus organ culture, although ICZ is less toxic than TCDD in this assay by a factor of 10^{-5} (35). Of special note in the context of the present investigation are the established activities of TCDD as an anti-initiator and as a promoter of carcinogenesis (36, 37). Similar cancer-related effects of ICZ or related oligomers may account for some of the cancer-modulating activities of I3C.

Because ICZ and TCDD may produce similar biological effects, it is of interest to compare a typical dietary dose of ICZ to the currently acceptable maximum dose of TCDD. Assuming that a 20% conversion of glucobrassicin to I3C occurs during maceration of plant material (2), a 100-g portion of brussels sprouts can provide between 10–50 μmol of I3C (38). A yield of 0.01% in the gastrointestinal tract, as indicated by the present studies, would provide a dose of 1–5 nmol (256–1280 ng) of ICZ. This dose is considerably in excess of the maximum acceptable daily human dose for TCDD established by the U.S. Environmental Protection Agency—i.e., 1.25 fmol (400 fg) for a 70-kg person. However, useful quantitative comparisons of the relative hazard, or benefit, of the two compounds as, for example, cancer modulators, cannot be made on the basis of available information. Affinity for the Ah-receptor protein *in vitro* and potency as an inducer of cytochrome P-450-dependent monooxygenases in cell culture may not be reliable indicators of results from long-term cancer tests in animals. Other factors to be considered include biological half-life, which may be as long as 10 yr for TCDD (39). The half-life of ICZ may be considerably shorter than this for several reasons, including the possibilities that ICZ may have less affinity for cellular storage sites than TCDD, ICZ may be metabolized to excretable products more rapidly than TCDD, and ICZ may be a better substrate for the multidrug resistance active-transport system than is TCDD (34). Although further studies are required to characterize the biological activity of ICZ, it appears unlikely that normal levels of ICZ from the diet are of significant hazard compared with the benefits of the micronutrients in *Brassica* vegetables (4).

We thank O. Hankinson, University of California, Los Angeles, for the gift of the Hepa 1c1c7 cells; L.-S. Kim, California Department of Public Health, Berkeley, CA, for HPLC-MS analyses; and J. Bergman, Royal Institute of Technology, Stockholm, Sweden, for an authentic sample of ICZ. C.B. is the recipient of a Junior Faculty Research Award (JFRA-303) from the American Cancer Society.

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4. I have been informed and believe that claims of the '477 application are subject to a rejection based on Liang Jin *et al.*, 1999, Cancer Research 59:3991-3997 (hereinafter "Liang") and also to a rejection based on Liang in view of U.S. Patent No. 6,001,868 (hereinafter "Firestone"), and U.S. Patent No. 5,981,568 (hereinafter "Kunz").

5. Attached hereto as Exhibit 1 is a copy of U.S. Patent No. 6,689,387. The filing date of this patent is September 23, 1999. I hereby confirm that the acts described in Section 13 of Exhibit 1 were carried out by me or at my direction in the United States of America prior to August 3, 1999.

6. As shown in Exhibit 1, I had treated a patient with cervical dysplasia by administering processed DIM (see col. 15, lines 15-39). Treatment with transdermal processed DIM for a period of two weeks, followed by two months of daily use of oral processed DIM, resulted in a more normal appearing cervix upon repeat pelvic examination by the same medical practitioner.

7. The patient was a 45 year old woman ("V.H.") with a long history of fibrocystic breasts, recurrent severe breast pain, and cervical dysplasia. The breast pain occurred on a monthly basis during the second half of the menstrual cycle and required the use of analgesics like ibuprofen. The breast pain diminished with onset of the menses. Abnormal pap smears of the uterine cervix were first noted in her mid-thirties. The cervical dysplasia was categorized as a "low grade squamous intraepithelial lesion" in a pretreatment pap smear (see paragraph 8 below). The patient began taking transdermal processed DIM in a 1.5% strength breast cream for relief of monthly breast pain. Dramatic resolution occurred over a period of 2 weeks. During this time, a reduction and disappearance of chronic vaginal discharge which had been present and attributed to the cervical dysplasia were also noted. Following two weeks of transdermal use of processed DIM, the patient began daily use of oral processed DIM (Indolplex™ from Bioresponse) at a dose of 50 mg per day of DIM. After two months of oral therapy, follow up pelvic examination revealed a more normal appearing cervix (see paragraph 9 below).

8. A cervical-vaginal pap smear, performed on the V.H. on July 6, 1998, before DIM use was initiated, showed "low grade squamous intraepithelial lesion" (see Exhibit 2). The presence of low grade squamous intraepithelial lesion (LGSIL) is consistent with human papillomavirus (HPV) infection and mild dysplasia. See Nguyen *et al.*, "The Bethesda System and Evaluation of Abnormal Pap Smears", Seminars in Surgical Oncology, 1999; 16:217-221, see Table 1 on page 218 (a copy of which is attached as Exhibit 3).

Subsequent to this test, the patent was treated with processed DIM as described above in paragraphs 5-7.

9. A follow up pap smear was performed on V.H. on August 3, 1999 (see Exhibit 4; see "Date Received"). The results of this test show that the abnormalities noted before DIM treatment were resolved. The diagnosis of "atypical squamous cells of undetermined significance" indicates that "some squamous cell abnormalities are more abnormal than those seen with reparative or inflammatory change, *but are not severe enough to qualify for dysplasia*" (emphasis added) (see Nguyen *supra* at 218).

10. Therefore, prior to the publication date of Liang, *i.e.*, prior to August 15, 1999, I had conceived and reduced to practice the treatment of cervical dysplasia in a human subject in need of such treatment with DIM.

11. I declare further that all statements made in this Declaration of my own knowledge are true and that all statements made on information and belief are believed to be true and further that these statements are made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the application or any patent issuing thereon.

Date: Sept 11, 2007 Michael A. Zeligs, M.D.